NUCLEAR TRANSFER PROMOTER FOR Rac PROTEIN AND METHOD OF SCREENING THE SAME

Technical Field

[0001]

The present invention is based on a finding that an isoprenoid synthesis inhibitor and/or a geranylgeranyl transferase inhibitor, particularly an HMG-CoA reductase inhibitor that is an isoprenoid synthase inhibitor has an action of promoting the transfer of Rac protein into a nucleus. That is, the present invention relates to a nuclear transfer promoter for Rac protein comprising an isoprenoid synthesis inhibitor and/or a geranylgeranyl transferase inhibitor, preferably an isoprenoid synthesis inhibitor, more preferably an HMG-CoA reductase inhibitor, use of the inhibitor as a nuclear transfer promoter, a method of promoting the transfer of Rac protein into a nucleus by using the inhibitor, and a pharmaceutical composition including the inhibitor. Further, the present invention relates to a blood vessel remedy including the nuclear transfer promoter for Rac protein as the active ingredient, and a method of screening a blood vessel remedy which comprises assaying the ability of Rac protein to transfer into a nucleus.

Background Art

[0002]

GTP-binding protein (G protein) is a generic name of endogenous protein group having an activity of hydrolyzing GTP,

and has been known for a G protein group involved in mRNA translation, a trimer G protein group conjugated with 7-times transmembrane receptor, a low-molecular-weight G protein group ("Experimental Medicine", 21:137-145, 2003) and the like. Among these groups, 100 or more kinds of low-molecular-weight G protein proteins have been reported as proteins having molecular weights of 20,000 to 30,000 with no subunit structure, and after isoprenylation, these proteins transfer to cell membranes to participate in intracellular signal transmission as GTP-bound form (on)/GDP-bound form (off).

[0003]

The low-molecular-weight G protein group is further divided into five super-families, that is, Ras, Rho, Rab, Arf and Ran (Physiol. Rev., 81:153-208, 2001). Among these families, the Rho family is further divided into subfamilies such as Rho, Rac, Cdc42. The Rho family regulates cellular functions via re-organization of an actin cytoskeleton, and similarly to the Ras family, participates in gene expression. Rho induces formation of actin stress fiber or focal contact, Rac induces formation of lamellipodia, and Cdc42 induces formation of filopodia.

[0004]

Specifically, Rac protein (also referred to as Rac1) is a protein consisting of about 200 amino acids and has a structure similar to that of Rho, but regulates an actin cytoskeleton in a mode different from that of Rho. Rac protein is also reported to activate JNK/p38 and NF-κB.

[0005]

On one hand, an HMG-CoA (3-hydroxy-3-methyl-glutaryl-CoA) reductase inhibitor is an inhibitor of an enzyme catalyzing conversion of HMG-CoA into mevalonic acid in an early rate-determining stage in biosynthesis of cholesterol, and is known as a hypercholesterolemia remedy. The HMG-CoA reductase inhibitor has been verified to reduce the onset of arteriosclerosis in a large-scale test, and from overlap analysis or the like, it has been revealed that this reduction of the onset is responsible for HMG-CoA reductase inhibitor's action in a vascular wall, aside from its action of reducing cholesterol by inhibiting HMG-CoA reductase in the liver.

[0006]

That is, it is believed that the HMG-CoA reductase inhibitor inhibits HMG-CoA reductase in cells of a vascular wall, and via its action of reducing the formation of isoprenoid, reduces the activity of low-molecular-weight G protein, thus exerting various influences on cellular functions to exhibit anti-inflammatory reaction in the vascular wall thereby suppressing arteriosclerosis.

[0007]

Further, the HMG-CoA reductase inhibitor has actions such as suppression of endothelial cell activation, improvement of endothelial functions, suppression or improvement of adhesion or foaming of monocytes/macrophages, suppression of migration/proliferation of smooth muscles, and stabilization of plaques, and Rho, Rac, and Cdc42 that are low-molecular-weight

G proteins in the Rho subfamily are reported to participate in these actions. Particularly, the effect of the HMG-CoA reductase inhibitor on improvement of endothelial functions appears evidently in a short time after administration, and is considered important among the various actions.

[0008]

Recently, it is revealed that Rac participates in signal transmission mediated by angiotensin II, PDGF, thrombin, endothelin, leukotriene B4 and the like in vascular walls and promotes the activity of NADPH, thus playing an important role in the progress of a vascular disease (Am. J. Physiol. Cell Physiol., 285:C723-734, 2003), and it is reported that Cdc42 also participates in proliferation of vascular endothelial cells and in recovery of barrier functions (J. Cell Sci., 114:1343-55, 2001; J. Biol. Chem., 277:4003-9., 2002; Circ. Res., 94:159-166, 2004) and also in signal transmission of endothelin (J. Biol. Chem., 278:29890-900, 2003).

[0009]

Further, the present inventors examined the influence of pitavastatin as HMG-CoA reductase inhibitor on gene expression in vascular endothelial cells, and they found that pitavastatin suppresses expression of inflammatory cytokine IL-8 or MCP-1, expression of endothelin and expression of PAI-1, promotes expression of NO synthase involved in vascular expansion and shrinkage, expression of thrombomodulin in a coagulation and fibrinolysis system (J. Atheroscler. Thromb., 9:178-183, 2002), and suppresses expression of PTX3 (promoting expression of TF

and serving as an indicator of progress of arteriosclerosis) (J. Atheroscler. Thromb., 11:62-183, 2004).

Disclosure of Invention

[0010]

As a result of eager study, the present inventors surprisingly found that Rac and Cdc42 were transferred to nuclei by treatment with an isoprenoid synthesis inhibitor and/or a geranylgeranyl transferase inhibitor, particularly by treatment with an HMG-CoA reductase inhibitor, and the present invention was thereby completed.

That is, the present invention relates to a nuclear transfer promoter for Rac protein comprising an isoprenoid synthesis inhibitor and/or a geranylgeranyl transferase inhibitor, preferably an isoprenoid synthesis inhibitor, more preferably one kind of isoprenoid synthesis inhibitor, that is, an HMG-CoA reductase inhibitor.

[0011]

The present invention also relates to use, as a nuclear transfer promoter for Rac protein, of an isoprenoid synthesis inhibitor and/or a geranylgeranyl transferase inhibitor, preferably an isoprenoid synthesis inhibitor, more preferably one kind of isoprenoid synthesis inhibitor, that is, an HMG-CoA reductase inhibitor, and provides a method of promoting the transfer of Rac protein into a nucleus, which includes administering an isoprenoid synthesis inhibitor and/or a geranylgeranyl transferase inhibitor to a cell.

[0012]

Further, the present invention provides a blood vessel remedy including the nuclear transfer promoter for Rac protein as the active ingredient, as well as a pharmaceutical composition for vascular treatment comprising the nuclear transfer promoter for Rac protein and a pharmaceutically acceptable carrier.

[0013]

Further, the present invention provides use of the nuclear transfer promoter for Rac protein in producing a blood vessel remedy, as well as a therapeutic/prevention method for vascular disorders including administering the nuclear transfer promoter for Rac protein in an effective amount for therapy/prevention to a patient in need of therapy/prevention of vascular disorders.

[0014]

Further, the present invention provides a method of screening a blood vessel remedy, which includes measuring the transfer of Rac protein into a nucleus. Specifically, the present invention provides a method of screening a blood vessel remedy, which includes adding a test substance to a Rac protein-expressing cell and measuring the transfer of Rac protein into the nucleus.

Brief Description of the Drawings

[0015]

Fig. 1 is a photograph, under a fluorescence microscope, of transformed cells which were introduced a gene encoding a green fluorescence protein (GFP) / Rac fusion protein, and were

cultured in the absence of pitavastatin.

Fig. 2 is a photograph, under a fluorescence microscope, of transformed cells which were introduced a gene encoding GFP/Rac fusion protein, and were cultured in the presence of pitavastatin.

Fig. 3 is a photograph, under a fluorescence microscope, of transformed cells whose nuclei were stained (red) by adding nucleus-staining dye Hoechst after introduction of a gene encoding GFP/Rac fusion protein and cultivation in the presence of pitavastatin.

Best Mode for Carrying out the Invention

[0016]

The present inventors measured the behavior of Rac protein in HUVEC with an HMG-CoA reductase inhibitor, particularly pitavastatin. For this measurement, a gene encoding a Rac /fluorescence protein GFP fusion protein was introduced into HUVEC to prepare a transformed cell expressing a GFP/Rac fusion protein. This transformed cell was cultured, and the state of Rac distributed in the cell in the presence or absence of pitavastatin was examined by observing the fluorescence of GFP. These results are shown in Figs. 1 to 3.

[0017]

Fig. 1 is a photograph substituted for a drawing, showing the result of observation, under a fluorescence microscope, of the state of Rac distributed in the transformed cells which has been cultured in the absence of pitavastatin. In Fig. 1, the

fluorescence of GFP can be observed in the nearly whole area of the cells, thus revealing that Rac protein region is distributed in the whole area of the transformed cells.

[0018]

Fig. 2 is a photograph substituted for a drawing, showing the result of observation, under a fluorescence microscope, of the state of Rac distributed in the transformed cells which has been cultured in the presence of pitavastatin. In Fig. 2, it can be observed that the fluorescence of GFP was localized in a certain area of the cells. For further confirming the position at which Rac is localized in the cells, the cells were stained with Hoechst (Lydon M., et al., J. Cell Physiol., 102, 175-181 (1980); SriramM., et al., Biochemistry, 31, 11823-11834 (1992)), and Fig. 3 is a photograph substituted for a drawing, showing the result of this staining. In Fig. 3, the nuclei stained with Hoechst are observed to be red, and this corresponded to the site at which the fluoresce of GFP is localized.

[0019]

Hoechst used herein is a fluorescent dye having an ability to permeate through a cell membrane and binding specifically to an AT sequence in a minor groove of DNA. From the above experiment, it has been revealed that upon treatment of the vascular endothelial cells with pitavastatin, GFP-Rac is transferred to a nucleus, that is, to the same position as the site stained with the nucleus-staining dye Hoechst. It has been thus revealed that the HMG-CoA reductase inhibitor has an action of allowing Rac protein in the cell to transfer into the nucleus.

[0020]

The nuclear transfer promoter for Rac protein according to the present invention exerts an important influence on the actions in which Rac protein is involved, such as regulation of cellular motility, cellular polarity, intracellular signal transmission and gene expression, particularly on regulation of gene expression in vascular wall cells, and is considered useful as a blood vessel remedy, particularly an endothelial cell function improver and a cell adhesion inhibitor.

[0021]

The isoprenoid synthesis inhibitor as the nuclear transfer promoter for Rac protein according to the present invention can include HMG-CoA synthase inhibitors (Proc. Natl. Acad. Sci. USA., 84:7488-92, 1987), HMG-CoA reductase inhibitors, AMPK activators such as fibrate (Biochem. Soc. Trans., 25:S676, 1997), and farnesylpyrophosphoric acid synthase inhibitors such as N-containing bisphosphonate. (Biochem. Biophys. Res. Commun., 264:108-111, 1999). The geranylgeranyl transferase inhibitor as the nuclear transfer promoter for Rac protein according to the present invention can include inhibitors described in known literatures, for example Biochemical Pharmacology, 60:1061-1068, 2000. These enzyme inhibitors may be any inhibitors capable of completely or partially inhibiting the activity of the objective enzyme.

[0022]

Specifically, the following compounds can be mentioned.

Lovastatin (chemical name:

(+)-(1S,3R,7S,8S,8aR)-1,2,3,7,8,8a-hexahydro-3,7-dimethyl-8-[2-[(2R,4R)-tetrahydro-4-hydroxy-6-oxo-2H-pyran-2-yl] ethyl]-1-naphthyl (S)-2-methyl butyrate (see US Patent No. 4,231,938));

Simvastatin (chemical name: (+)-(1S,3R,7S,8S,8aR)-1,2,3,7,8,8a-hexahydro-3,7-dimethyl-8-[2-[(2R,4R)-tetrahydro-4-hydroxy-6-oxo-2H-pyran-2-yl] ethyl]-1-naphthyl 2,2-dimethyl butanoate (see US Patent No. 4,444,784));

Pravastatin (chemical name: (+)-(3R,5R)-3,5-dihydroxy-7-[(1S,2S,6S,8S,8aR)-6-hydroxy-2-methyl-8-[(S)-2-methylbutyryloxy]-1,2,6,7,8,8a-hexahydro-1-naphthyl] heptenoic acid (see US Patent No. 4,346,227));

Fluvastatin (chemical name: (3RS,5SR,6E)-7-[3-(4-fluorophenyl)-1-(1-methylethyl)-1H-indol-2-yl]-3,5-dihydroxy-6-heptenoic acid (see US Patent No. 5,354,772));

Atorvastatin (chemical name: (3R,5R)-7-[2-(4-fluorophenyl)-5-isopropyl-3-phenyl-4-phenyl carbamoyl-1H-pyrol-1-yl]-3,5-dihydroxyheptanoic acid (see US Patent No. 5,273,995));

Cerivastatin (chemical name: (3R,5S)-erythro-(E)-7-[4-(4-fluorophenyl)-2,6-diisopropyl-5-methoxymethyl-pyridin-3-yl]-3,5-dihydroxy-6-heptenoic acid (see US Patent No. 5,177,080));

Mevastatin (chemical name: (+)-(1S,3R,7S,8S,8aR)-1,2,3,7,8,8a-hexahydro-7-methyl-8-[2-

[(2R,4R)-tetrahydro-4-hydroxy-6-oxo-2H-pyran-2-yl] ethyl]-1-naphthyl (S)-2-methyl butyrate (see US Patent No. 3,983,140));

Rosuvastatin (chemical name: 7-[4-(4-fluorophenyl)-6-isopropyl-2-(N-methyl-N-methane sulfonylaminopyridine)-5-yl]-(3R,5S)-dihydroxy-(E)-6-heptenoic acid (see US Patent No. 5,260,440 and Japanese Patent No. 2,648,897)); and

Pitavastatin ((3R,5S,6E)-7-[2-cylopropyl-4-(4-fluorophenyl)-3-quinolyl]-3,5-dihydroxy-6-heptenoic acid (see US Patent No. 5,856,336 and Japanese Patent No. 2,569,746)).

If pharmaceutically necessary, the inhibitor can be used as a salt or solvate. Particularly preferable inhibitor is pitavastatin.

[0023]

In another aspect of the present invention, the method of screening a blood vessel remedy, which includes measuring the transfer of Rac protein into the nucleus includes a method of labeling or staining Rac protein and identifying the transfer thereof into the nucleus. The method of labeling Rac protein includes genetic engineering techniques. Specific examples include method of utilizing the fusion protein of fluorescence proteins BFP, CFP and YFP, including GFP (Atsushi Miyawaki: Intracellular phenomenon is visualized by fluorescence bio-imaging. Riken News 255, September 2002) and Rac protein. The method of staining Rac protein includes immunological

techniques. Specifically, use of a fluorescence antibody or enzyme antibody can be mentioned. The method is particularly preferably a method which involves preparing a fusion protein of a fluorescence protein such as GFP and Rac protein and then visually identifying the transfer of the fusion protein into the nucleus.

[0024]

The nuclear transfer promoter for Rac protein according to the present invention can be used not only as a pharmaceutical preparation in therapy/prevention of vascular disorders, but also as a reagent for localizing Rac protein in cellular nuclei in a test using various cells. That is, the nuclear transfer promoter can be used not only as the active ingredient in a pharmaceutical preparation but also as an experimental reagent or a reagent in a diagnostic medicine.

[0025]

The blood vessel remedy of the present invention includes a pharmaceutical composition for vascular treatment, which uses the nuclear transfer promoter for Rac protein according to the present invention or is made of the nuclear transfer promoter and a pharmaceutically acceptable carrier.

[0026]

The route of administration of the blood vessel remedy of the present invention includes, for example, oral administration by tablets, capsules, granules, powders, syrups or the like and parenteral administrations by intravenous injections, intramuscular injections, suppositories,

inhalations, transdermal absorbers, eye-drops, nasal agents or the like.

For preparing the pharmaceutical preparation in such various forms, the active ingredient is used alone or in suitable combination with other pharmaceutically acceptable one or more additives such as excipient, binder, extender, disintegrating agent, surfactant, lubricant, dispersant, buffer agent, preservative, taste corrective, flavoring, coating agent, carrier, diluent or the like.

[0027]

Particularly, the route of administration of the HMG-CoA reductase inhibitor is preferably oral administration. For preparation of the pharmaceutical preparation for oral administration, the pH of the preparation is regulated in consideration of the stability of the active ingredient (Japanese Patent Application Laid-open No. 2-6406, Japanese Patent No. 2,774,073, and WO97/23200, the disclosure of which is incorporated by reference herein).

[0028]

The amount of the pharmaceutical preparation administered varies depending on the weight, age, sex and symptoms of the patient, and in the case of an adult, it is usually preferable that the isoprenoid synthesis inhibitor and/or the geranylgeranyl transferase inhibitor as the active ingredient is administered orally in a daily dose of 0.01 to 1000 mg, particularly 0.1 to 100 mg, all at once or in divided portions.

[0029]

Example

Hereinafter, the present invention is described in more detail by referenced to the Examples, but the present invention is not limited to the Example.

[0030]

Example 1

A gene encoding the whole area of a Rac translation region was introduced into a predetermined position of a commercial plasmid pEGFP-C1 for preparation of a fusion protein consisting of GFP and a desired protein, to construct a plasmid comprising the GFP-Rac gene.

After HUVECs were put to a 6-well plate and then cultured overnight in EGM-2 medium. Using Fugene 6, the plasmid construct DNA prepared above was added in an amount of 0.8 μg/well to the cells. The cells were further cultured for 21 hours in EGM-2 medium, and then the fluorescence of GFP was observed under a fluorescence microscope. The result of this observation is shown in Fig. 1.

Six hours after the culture of HUVECs to which the plasmid construct DNA had been added was initiated, pitavastatin was added to the cells to a final concentration of 1 μM. And then the cells were subjected to stationary culture for 15 hours, fixed onto a prepared slide and observed under a fluorescence microscope. The result of this observation is shown in Fig. 2. Further, the cells were stained with the nucleus-staining dye Hoechst. The result of this staining is shown in Fig. 3.

Industrial Applicability

[0031]

The present invention relates to a nuclear transfer promoter for Rac protein, and Rac protein is known to participate in proliferation of vascular endothelial cells, in recovery of barrier functions, and in signal transmission of endothelin and plays an important role in the progress of vascular diseases via participation in regulating the expression of various genes involved in vascular shrinkage/expansion, inflammations, and blood coagulation/fibrinolysis, and thus the medicine of the present invention is industrially extremely useful as a pharmaceutical preparation for treatment and prevention of various vascular diseases.

In addition, the present invention provides a method of screening a blood vessel remedy which includes measuring the transfer of Rac protein to the nucleus, and is industrially useful as a means for developing new therapeutic agents and prevention agents for vascular diseases.